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Serine Hydroxymethyltransferase from Soybean Root Nodules[1]

PURIFICATION AND KINETIC PROPERTIES

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ABSTRACT

Serine hydroxymethyltransferase has been purified 1,550-fold from the plant fraction of soybean (*Glycine max* [L]. Merr. cv Williams) nodules. The pH optimum for the enzyme was at 8.5. The native molecular weight was 230,000 with a subunit molecular weight of 55,000 which suggested a tetramer of identical subunits. The enzyme kinetics for the enzyme were Michaelis-Menten; there was no evidence for cooperativity in the binding of either substrates or product inhibitors. There were two K_m values for serine at 1.5 and 40 millimolar. The K_m for *l*-tetrahydrofolate was 0.25 millimolar. *l*-Methyl-, *l*-methenyl-, and *l*-methylene-tetrahydrofolates were all noncompetitive inhibitors with *l*-tetrahydrofolate with K_i values of 1.8, 3.0, and 2.9 millimolar, respectively. Glycine was a competitive inhibitor with serine with a K_i value of 3.0 millimolar. The intersecting nature of the double reciprocal plots together with the product inhibition data suggested an ordered mechanism with serine the first substrate to bind and glycine the last product released. The enzyme was insensitive to a wide range of metabolites which have previously been reported to affect its activity. These results are discussed with respect to the roles of serine hydroxymethyltransferase and the one-carbon metabolite pool in control of the carbon flow to the purine biosynthetic pathway in ureide biogenesis.

The ureides, allantoin and allantoic acid, are the major forms of nitrogen transported from the nodules of nitrogen-fixing soybeans and cowpeas (10) accounting for 78% of the total nitrogen on a seasonal basis. In these plants, ammonia, produced by the bacteroids, is initially assimilated into glutamine and aspartate and subsequently incorporated into the purine ring via *de novo* purine biosynthesis (3, 22). Partial oxidation of the purine ring gives rise to allantoic acid (22).

Glycine is a requirement for purine synthesis and current results suggest that the main source in the nodule is from serine cleavage by serine hydroxymethyltransferase (EC 2.1.2.1) which catalyzes the reaction:

$$\text{Serine} + FH_4 = \text{glycine} + \text{methylene}\cdot FH_4.$$

This reaction also yields methylene·FH_4 which can then be oxidized to methenyl·FH_4,[2] another purine biosynthesis intermediate. Timecourse studies of soybean nodule development (23) and comparative studies between the nodules of amide- and ureide-transporting plants (20) have implicated the involvement of SHM in ureide biogenesis. Localization studies have shown the enzyme to be present in the proplastid fraction of the nodule (2) and synthesis of [^{14}C]XMP has been achieved using [3-^{14}C]-serine and purine biosynthesis substrates in isolated proplastids from soybean nodules (3).

The SHM has been purified and its kinetic properties examined from several sources including liver from monkey (18), rat (14), and rabbit (8), cauliflower flower primordia (13), tobacco (16), and mung bean seedlings (19). The enzyme is of particular interest in soybean nodules as it occupies a potentially important position in the carbon flow of the nodule. This paper reports the purification and kinetic properties of SHM from soybean root nodules and discusses the role of the enzyme in ureide biogenesis.

MATERIALS AND METHODS

Materials. Soybean plants (*Glycine max* (L.) Merr. cv Williams) inoculated with *Rhizobium japonicum*, strain 3I1b 142, were grown in a controlled environment growth cabinet with a daylength of 16 h and a day/night temperature regime of 28°C/24°C (23).

Biochemicals were obtained from Sigma. [3-^{14}C]Serine was from Amersham, and the enzyme preparations and standard proteins used in M_r determinations and substrate estimations were all from Sigma. Scintillation cocktails were obtained from Research Products International. Affi-Gel blue was from Bio-Rad and all other chromatographic materials were from Sigma. Methenyltetrafolate was synthesized from N^5-formyltetrahydrofolate according to the method of Scott (26).

Enzyme Assays. All assays were performed at 25°C. The concentration of *l*-tetrahydrofolate used in kinetic experiments was determined enzymically using formimino-L-glutamate transferase (EC 2.1.2.5) and formiminotetrahydrofolate cyclodeaminase (EC 4.3.1.4). The 5,10-methenyl·FH_4 formed by these coupled reactions was measured at 365 nm and was proportional to the concentration of *l*-tetrahydrofolate.

The SHM was assayed by measuring the transfer of radiolabel from [3-^{14}C]serine to FH_4 using a modification of the method of Taylor and Weissbach (27). The assay mixture contained 50 mM

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[2] Abbreviations: demedone, 5,5-dimethyl-1,3-cyclohexanedione; FH_4, *l*-tetrahydrolate (tetrahydropteroylglutamate); methylene·FH_4, *l*-N^5, N^{10}-methylenetetrahydrofolate; methenyl·FH_4, *l*-N^5, N^{10}-methenyltetrahydrofolate; methyl·FH_4, *l*-N^5-methyltetrahydrofolate; SHM, serine hydroxymethyltransferase; ACES, *N*-2-acetamido-2-aminoethanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid.

Tris-HCl (pH 8.5), 10 mM 2-mercaptoethanol, serine, and FH_4 at appropriate concentrations, and 0.4 μCi of [3-^{14}C]serine. The reaction (final volume, 0.4 ml) was started by the addition of FH_4 and stopped by the addition of 0.3 ml of 1.0 M acetic acid. Formaldehyde (0.2 ml of 0.1 M) and dimedone (0.3 ml of 0.4 M) were added and the mixture boiled for 5 min. After cooling, 5 ml toluene were added and the two phases separated by centrifuging; 2.5 ml were removed from the upper phase, added to 3 ml of 3a20 counting cocktail, and counted by liquid scintillation spectrophotometry. It is important to note that enzyme assay mixtures were not preincubated in the presence of FH_4. This has been reported to give rise to assay artifacts due to the instability of FH_4 in the assay solution (25).

Urease was assayed radiochemically by trapping the $^{14}CO_2$ released from [U-^{14}C]urea (9). Aspartate aminotransferase was assayed as described in Farnden and Robertson (7). Pyruvate kinase and malate dehydrogenase were assayed as has been done previously (24).

Enzyme Purification. All purification steps, except the Affi-Gel blue affinity step which was performed at room temperature, were carried out at 4°C. Nodules were harvested 26 d after inoculation and crushed in 1:1 (w/v) 50 mM Tricine-KOH (pH 8.0), containing 0.4 M sucrose, 1 mM $MgCl_2$, 20 mM DTE, and 5 mM glutathione.

The macerate was squeezed through three layers of cheese cloth and centrifuged at 20,000*g* for 25 min to yield the crude plant fraction of the nodule. This supernatant preparation was fractionated by $(NH_4)_2SO_4$ precipitation. The fraction precipitating between 40 and 60% $(NH_4)_2SO_4$ was collected by centrifugation and resuspended in 3 ml of 25 mM Tris-HCl, pH 8.5, containing 5 mM DTE and 50 μg/ml pyridoxal phosphate.

The solution was then applied to a Sepharose CL-6B column (45 × 3.0 cm), which had been equilibrated with the $(NH_4)_2SO_4$ resuspension buffer. Fractions containing peak activity were pooled and made 10% with respect to glycerol and 4 mM with respect to serine.

The enzyme solution was then stirred with Affi-Gel blue previously equilibrated with 25 mM Tris-HCl (pH 8.5), containing 5 mM DTE, 50 μg/ml pyridoxal phosphate, 4 mM serine, and 10% (v/v) glycerol (AGB buffer), for 30 min at 4°C. This mixture was poured into a column (5 × 1.0 cm) and unbound protein eluted with AGB buffer. The column was then washed with 25 ml of AGB buffer containing 10 mM NAD^+. SHM was then eluted with 15 ml of AGB buffer containing 500 mM KCl.

The enzyme solution was then desalted using a Sephadex G-25 column (20 × 1.5 cm) equilibrated with AGB buffer. Fractions (10 ml) were collected at a flow rate of 65 ml/h, and those containing SHM activity were pooled.

The desalted SHM solution was applied to a DEAE-cellulose column (17 × 0.7 cm) previously equilibrated with 25 mM Tris-HCl (pH 8.5), containing 5 mM DTE, 4 mM serine, and 10% (v/v) glycerol. Pyridoxal phosphate (100 μl of a 500 μg/ml solution) was added to the collection tubes, and fractions (4.5 ml) were collected by elution with a linear salt gradient from 0 to 250 mM NaCl in a total volume of 150 ml of the buffer used to equilibrate the column, at a flow rate of 30 ml/h. SHM activity eluted from this column at 135 mM NaCl.

Protein concentrations were measured by the method of Bradford (4). Tube gels, 7.5% polyacrylamide (1), were prepared with 0.1 M Tris-0.1 M glycine buffer, pH 9.0, which was also used in the electrophoresis resevoirs. Bromophenol blue was used as a marker dye. Gels were stained for protein using Coomassie blue G-250.

Molecular Weight Determination. A 45 × 3 cm column of Sepharose CL-6B was equilibrated with 25 mM Tris-HCl (pH 8.5), 0.1 M KCl, 5 mM DTE, 50 μg/ml pyridoxal phosphate, and 4 mM serine. The sample was applied to the column in a volume of 1.0 ml, eluted with the above buffer, and 3.5 ml fractions were collected at a flow rate of 35 ml/h. Aspartate aminotransferase (M_r 110,000), pyruvate kinase (M_r 240,000), malate dehydrogenase (M_r 71,000), and urease (M_r 480,000 were used as standard proteins. SHM and the standard proteins were located by enzyme assay.

Subunit mol wt was determined using SDS-gel electrophoresis (24). BSA (M_r 66,000), egg albumin (M_r 45,000), carbonic anhydrase (M_r 29,000), phosphorylase *b* (M_r 97,400), and β-galactosidase (M_r 116,000) were used as protein standards.

Kinetic Analyses. All kinetic experiments were performed at least twice and gave similar results. In the figures all points are the means of replicates. For the calculations of kinetic constants the method of Wilkinson (28) was used on all data points.

RESULTS AND DISCUSSION

Enzyme Purification. A summary of the purification scheme for SHM from the plant fraction of soybean nodules is shown in Table I. A 1477-fold purification of SHM activity was achieved. The purified enzyme could be stored for 8 weeks at −20°C with no loss in activity. At the end of this purification procedure one protein band only was visible on 7.5% polyacrylamide gels (data not shown). Assay of 1 mm gel slices for SHM showed enzyme activity and the protein band to be coincident on the gel.

The addition of 4 mM serine to the pooled Sepharose 6B fractions was necessary to prevent total inactivation of the enzyme upon exposure to KCl, which was used in the next chromatographic step. Once exposed to NaCl or KCl in the absence of serine, enzyme activity could not be restored. Whether or not this was a specific monovalent cation effect was not investigated further.

Initial elution of the Affi-Gel blue column with 10 mM NAD^+ removed contaminating dehydrogenase enzymes which also bind to this affinity gel.

The exceptionally high recovery of enzyme was attributed to the presence of pyridoxal phosphate throughout the purification and, to the inclusion of 4 mM serine and 10% (v/v) glycerol in the buffers after Sepharose CL-6B chromatography. Stabilization of SHM by serine was reported by Rao and Appaji Rao (19) in mung bean seedlings, however these authors did not use substrate stabilization in their purification but relied on the presence of 2-mercaptoethanol and pyridoxal phosphate.

The possibility also exists that there may have been considerable masking of SHM activity in the crude plant fraction. This could be caused by the presence of inhibitory substances or by uncontrolled interferences in enzyme assay. No investigation was carried out to differentiate between these possibilities.

The apparent increase in activity between the $(NH_4)_2SO_4$ fraction and Sepharose CL-6B chromatography steps (Table I) could reflect the removal of an $(NH_4)_2SO_4$ effect on the enzyme.

Physical Properties. *Molecular Weight Determination.* The mol wt of SHM was determined as 230,000 ± 30,000 using molecular exclusion chromatography on Sepharose CL-6B (Fig. 1A). This is comparable to values reported from mammalian and bacterial sources (8, 12, 14, 18). The enzyme purified from mung bean seedlings (19) had a mol wt of 220,000. The subunit mol wt was 55,000 ± 1,000 (Fig. 1B). Only one band was visible on both 7.5 and 10% polyacrylamide gels containing 0.1% SDS. These results indicated that SHM from soybean nodules is composed of four identical subunits, and is in agreement with results reported for the enzyme from other sources (18, 19).

Dependence on pH. SHM showed an optimum for V_{max} at pH 8.5 (Fig. 2). Similar pH optima have been reported in other plant tissues (19, 29) and bacteria (30). Animal tissue exhibited a lower optimum at pH 7.4 From these data a theoretical V_{max} at pH 8.5 was calculated to be 1,712 μmol min^{-1} mg^{-1} protein. No information with respect to possible ionizable groups on the enzyme

Table I. *Purification of Serine Hydroxymethylase from Soybean Root Nodules*

	Total Protein	Total Activity[a]	Specific Activity	Pure	Recovery
	mg	*nmol min^{-1}*	*nmol min^{-1} mg^{-1}*	*-fold*	*%*
Crude extract	144	1,160	8.1	1	100
$(NH_4)_2SO_4$, 40-60%	27.5	843	31	4	73
Sepharose CL-6B	4.3	1,368	318	39	118
Affi-Gel blue	0.128	804	6,385	788	69
DEAE-Cellulose	0.093	1,113	11,968	1,477	96

[a] Enzyme activity was measured by determining N^5,N^{10}-[^{14}C]methylene·FH_4 production from [3-^{14}C]serine.

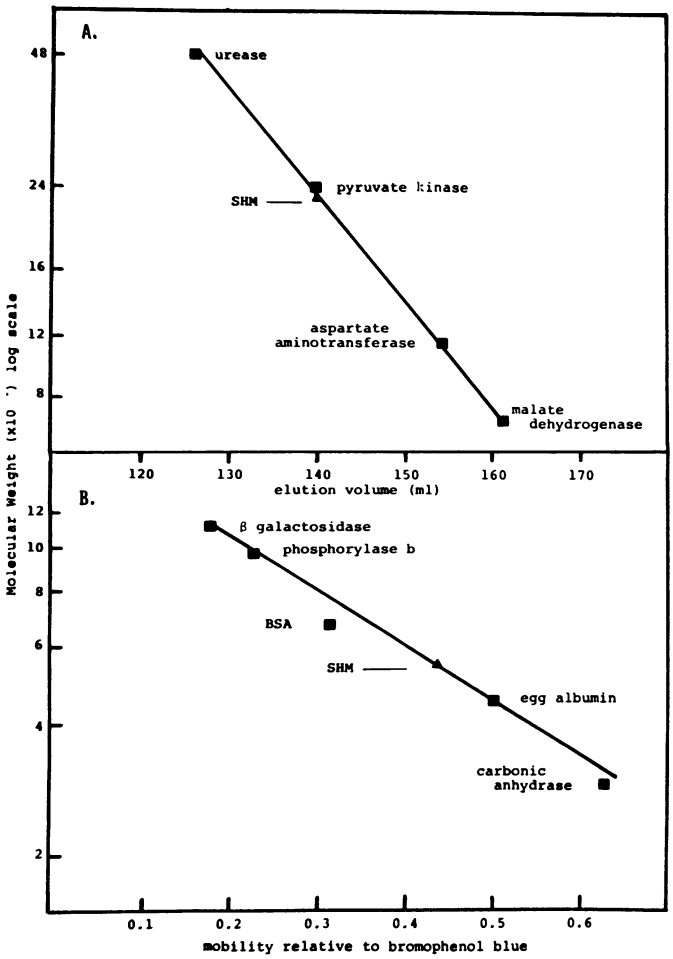

FIG. 1. A, Determination of the mol wt of SHM using Sepharose CL-6B column chromatography. The details of the procedure are given under "Materials and Methods." B, Determination of the subunit mol wt of SHM. SDS-PAGE was as described under "Materials and Methods." Standard proteins (■), SHM (▲). Both lines were fitted by regression using least squares.

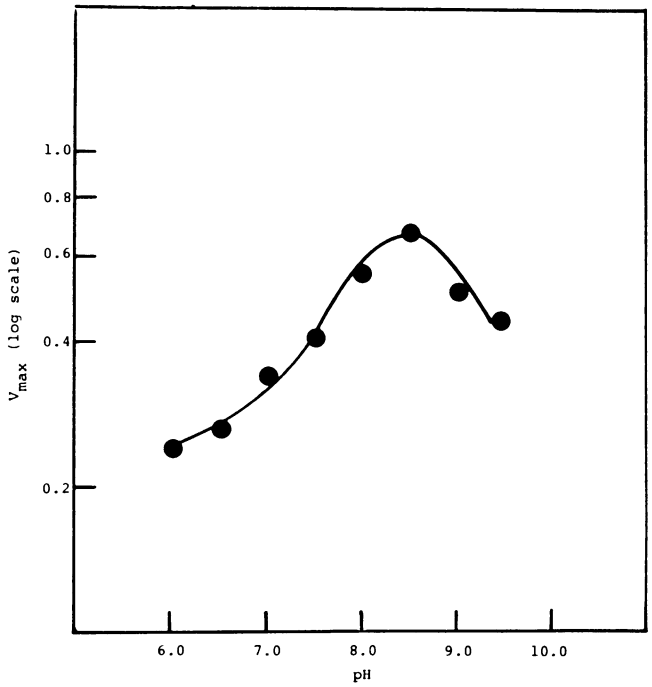

FIG. 2. The effect of pH on velocity for SHM. pH was varied over the range 6.0 to 9.5; 50 mM ACES was used as the buffer over the range pH 6.0 to 7.5, 50 mM Tris-HCl from pH 7.5 to 9.0, and CHES[2] for pH 9.0 and 9.5. At saturating FH_4 concentration (1.75 mM), serine was varied from 0.67 to 4.0 mM. The Y-intercept of double reciprocal plots (1/V *versus* 1/[S]) gave the V_{max} values. The optimum value for V_{max} at pH 8.5, was 1712 μmol min^{-1} mg^{-1} protein.

important in the reaction could be interpolated from these data. No enzyme activity could be detected above pH 9.5. The pK value for the 2-amino group of serine is 9.15 and could account for this loss in activity.

Kinetic Properties. *Determination of K_m Values.* Serine and FH_4 showed K_m values of 1.5 and 0.25 mM, respectively (Fig. 3). A second K_m (data not shown) was observed for serine at 40 mM. The enzyme from mung bean seedlings (19) also demonstrated two K_m values for serine. The intersecting pattern of the Lineweaver-Burk plots (Fig. 3) are consistent with both substrates binding before there is any product release. In contrast to the kinetics of SHM from both monkey liver (18) and mung bean seedlings (19), the enzyme from soybean nodules exhibited Michaelis-Menten kinetics. There was no evidence for cooperativity in substrate binding. Slope replots of the initial rates data (Fig. 3) were linear, compatible with this lack of regulatory properties. Care was taken to avoid heat treatments which have been suggested to destroy the allosteric properties of SHM from other sources (18). Ramesh and Appaji Rao (17), in work with SHM from monkey liver, reported that concentrations of serine above 3.6 mM totally abolished cooperativity in FH_4-binding. The purification procedure adopted for the soybean nodule enzyme utilized serine substrate stabilization at 4 mM (see above). However, enzyme which had not been stabilized by the addition of serine similarly showed no evidence for cooperativity in substrate binding. Furthermore, as the concentration of serine in the plant fraction of the nodule is of the order of 4.25 mM (using a value of 1.7 μmol serine/g nodules (20) and a nodule plant fraction volume of 0.4 ml/g (11), it seems unlikely that SHM would exhibit cooperative effects in substrate binding in soybean nodules under physiological conditions.

Schirch and Quashnock (25) have suggested that the apparent cooperativity in FH_4 binding observed with the monkey liver enzyme (17) could be caused by loss of FH_4 during the assay period due to oxidation by dissolved molecular oxygen. In con-

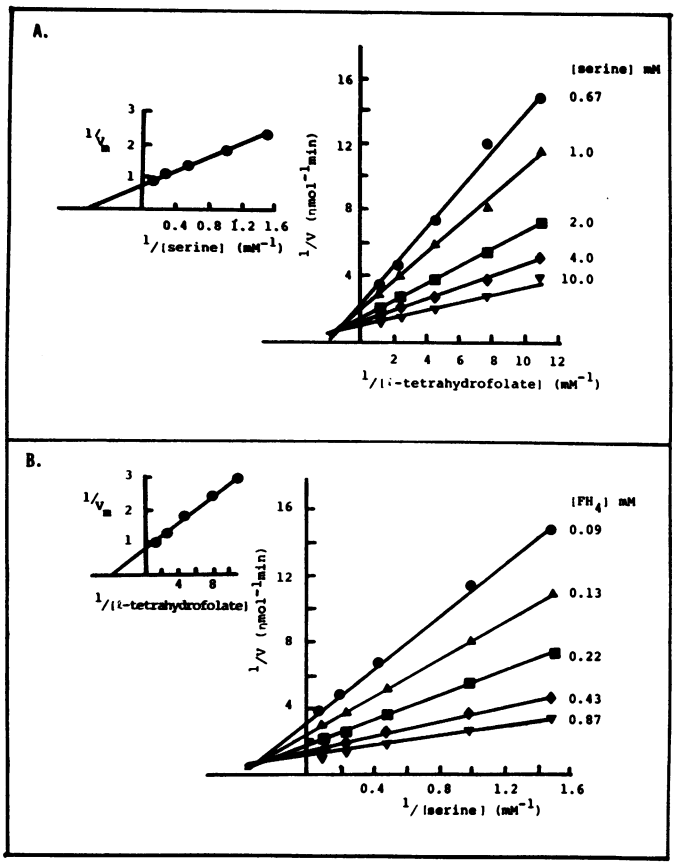

FIG. 3. Determination of K_m values for serine and *l*-tetrahydrofolate. A, Reciprocal plots of initial velocity *versus* FH_4 concentration at changing fixed serine concentrations. The inset shows a secondary plot of the Y-intercepts versus the reciprocal concentration of serine to give K_m (serine). B, The data in (A) replotted to obtain K_m (*l*-tetrahydrofolate).

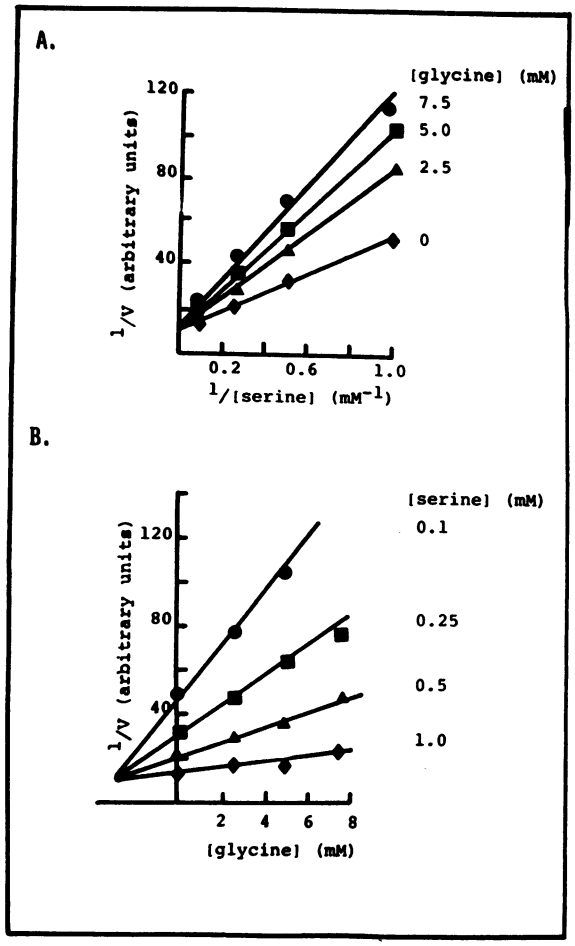

FIG. 4. A, Competitive inhibition by glycine with serine. At constant FH_4 concentration (0.31 mM) and changing fixed serine concentrations (1.0 mM to 10 mM), the glycine concentration was varied from 0 to 7.5 mM. A replot (B) according to Dixon (6) gave an estimate of the K_i value.

trast to the studies of the monkey liver and mung bean enzymes (17, 19), the SHM assay in this study did not involve preincubation with FH_4 and was for a shorter time period.

Inhibition Studies. Product inhibition by glycine was competitive with serine with a K_i value of 3.0 mM (Fig. 4, Table II). This, together with the intersecting nature of the Lineweaver-Burk plots (Fig. 3) is consistent with an ordered reaction mechanism with serine the first substrate to bind and glycine the last product released. Methenyl·FH_4 and methyl·FH_4 were both noncompetitive inhibitors with FH_4 and had K_i values of 3.0 and 1.8 mM, respectively (Table II). Estimation of a K_i value for methylene·FH_4 was complicated by the presence of contaminating FH_4 in the synthesized product. However, the inhibition pattern was clearly noncompetitive with FH_4 and the K_i was estimated to be 2.9 mM (Table II). The K_i values obtained for all three one-carbon derivatives of tetrahydrofolate were significantly higher than the K_m for FH_4. Although no K_m values were determined for the enzyme in the direction of serine synthesis, the reaction is known to be reversible (5), and the similarity of the K_i for glycine with the K_m for serine would support this.

Rao and Appaji Rao (19) showed NADH to be an allosteric positive effector and NAD^+ an allosteric negative effector with the enzyme from mung bean cotyledons. These metabolites had little or no effect on the soybean nodule enzyme, and this enzyme was also unaffected by methionine, *S*-adenosylmethionine, XMP, or IMP. Phosphoglycerate, a precursor metabolite in the proposed serine synthetic pathway in soybean nodules (Fig. 5) (2), also had no effect on SHM activity. These inhibition data suggest that SHM is not a regulatory enzyme in soybean nodules.

Table II. *Kinetic Constants and Properties of SHM*

K_m values	Serine	1.5 mM, 40 mM
	l-Tetrahydrofolate	0.25 mM
K_i values	Glycine (competitive with serine)	3.0 mM
	Methenyl·FH_4 (noncompetitive with FH_4)	3.0 mM
	Methyl·FH_4 (noncompetitive with FH_4)	1.8 mM
	Methylene·FH_4 (noncompetitive with FH_4)	2.9 mM
Physical properties	Native mol wt	230,000
	Subunit mol wt	55,000
	Optimum pH	8.5

This is in contrast to the enzyme purified from mung bean which has been suggested to be allosterically regulated (19).

SHM in Ureide Biogenesis. SHM occupies a central position in the flow of carbon from photosynthate to the purine ring in the synthesis of ureides (Fig. 5) (2, 22). Through its products, glycine and methylene.FH_4, this enzyme has the potential to account for all four carbons in the allantoate molecule. Glycine is totally incorporated into the purine ring by glycinamide ribonucleotide synthetase and accounts for two carbons. Glycinamide ribonucleotide synthetase activity has been measured in soybean nodules, associated with 5-phosphoribosylpyrophosphate amidotransferase (PHS Reynolds, unpublished data). The

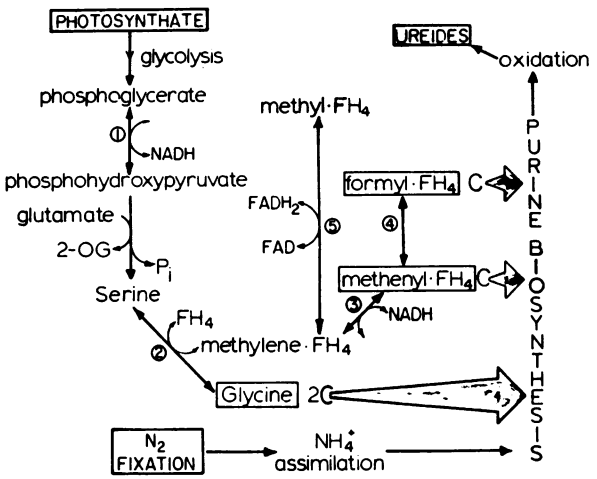

FIG. 5. Central role of SHM and one-carbon metabolism in ureide biogenesis. 1, Phosphoglycerate dehydrogenase (EC 1.1.1.95); 2, SHM (EC 2.12.1); 3, methylene·FH_4 dehydrogenase (EC 1.5.1.15); 4, methyl·FH_4 cyclohydrolase (EC 3.5.4.9); 5, methylene·FH_4 reductase (EC 1.1.1.68).

other two carbons come from the 1-C transfer system via methenyl·FH_4 formyl·FH_4.

In contrast to the enzyme from mung bean cotyledons and monkey liver (18, 19) the enzyme from soybean nodules is clearly not allosterically regulated. Indeed, it has been suggested (25) that the apparent allosteric properties of the monkey liver and mung bean enzymes relate to artifacts of the assay system. In view of the similarity of the K_m and K_i values for serine and glycine, and the fact that nodule concentrations for these metabolites are higher than those values (4.3 and 6.3 mM, respectively), it is possible that metabolites of the one-carbon pool may be important in determining flux through this reaction. The fact that the K_m value for FH_4 is significantly lower than the K_i values shown by any of the one carbon-FH_4 compounds, together with the existence of a second higher K_m for serine, may favor the reaction proceeding in the direction of glycine and methylene.FH_4 synthesis. Fujioka (8), in his study of the enzyme from rat liver, found the reaction was favored in the direction of glycine synthesis with the ratio glycine synthesis/glycine utilization being 1.8.

Both methylene·FH_4 dehydrogenase and methenyl·FH_4 cyclohydrolase have been demonstrated in soybean nodules (23). Methylene·FH_4 dehydrogenase has been demonstrated in the proplastid fraction of soybean nodules, along with SHM and other enzymes of purine biosynthesis (2). Methyl·FH_4 has been implicated as an important 1-C-storage compound in plants (5), however there has been no kinetic study of methylene·FH_4 reductase in soybean nodules. There have been thorough investigations of the control of N flow in nodules with glutamine synthetase (11) and 5-phosphoribosylpyrophosphate amidotransferase (21) having important regulatory roles. ATP levels have been shown to be important in the supply of OAA to ammonia assimilation by controlling the relative activities of PEP carboxylase and pyruvate kinase (15). There is no doubt that 1-C metabolism is more active in ureide-transporting as compared with amide-transporting nodules, as represented by the higher levels of methionine and the aromatic amino acids in ureide-transporting nodules (20).

It will therefore be of interest to investigate the enzymes of 1-C metabolism in soybean nodules and to establish the relative amounts of the 1C-FH_4 compounds and their possible sublocalization within the nodule cell, with a view to reaching an understanding of the control of C flow to purine biosynthesis in the production of ureides.

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